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13. ABSTRACT *(Maximum 200 Words)*
Chromium(VI) reduction by human lung is similar to that in liver, with significant rates of reduction using chromium(VI) concentrations anticipated for occupational exposure. The lung results are consistent with a prominent role for cytochrome b_5 in cooperation with NADPH:P450 reductase or NADH:b_5 reductase as principal mediators of Cr(VI) reduction. There is little interindividual difference in Cr(VI) reduction catalyzed by lung microsomes. During Cr(VI) reduction, the reactive intermediate Cr(V) appears early and is generated over a long period of time. Cr(IV) and Cr(III) appear later in the time courses, suggesting a series of consecutive one-electron reductions: Cr(VI) → Cr(V) → Cr(IV) → Cr(III). Similar to iron, small amounts of certain redox-active quinones can markedly stimulate microsomal Cr(VI) reduction, causing a more rapid reduction of both Cr(VI) and Cr(V). Individuals who are simultaneously exposed to chromium and quinones, which are ubiquitous environmental pollutants, are therefore likely at greater risk for chromium toxicity. The effect of quinones under aerobic conditions was influenced by the quinone itself, the quinone concentration, and the ratio of O_2 to Cr(VI). Other redox active compounds (methyl viologen, azobenzene, and 2,4-dinitrophenylhydrazine) cause little or no stimulation of Cr(VI) reduction. Human cytochrome P450s do not contribute to the quinone effect; the quinones are likely reduced by cytochrome b_5 in cooperation with NADPH:P450 reductase or NADH:b_5 reductase.

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Final Technical Report (Cumulative for 1 April 1998 – 28 February 1999)
AFOSR Grant no. F49620-98-1-0079
CHROMIUM TOXICITY: REDUCTIVE ACTIVATION BY HUMAN ENZYMES
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ORIGINAL OBJECTIVES:

The reductive metabolism of Cr(VI) to Cr(III), via reactive intermediates, is thought to play a key role in the cytotoxicity, mutagenicity, and carcinogenicity of Cr compounds. While several studies describe the reductive activation of Cr(VI) in rodents, there are significant differences between the Cr-reducing enzymes in humans and rodents. Preliminary data also suggest that iron can stimulate the rate of Cr(VI) reduction in human liver and lung. All of the data imply a significant role for human enzymes in the reductive activation of Cr(VI), and exemplify the need to study the problem using human tissue.

Cr(VI) reduction may result in the generation of several reactive species (e.g. Cr(V), Cr(IV), reactive oxygen species (ROS)), which likely contribute to various types of damage including Cr-DNA adducts, DNA strand breaks, and oxidation of DNA and lipids. Different reductants can yield different types and levels of reactive intermediates and hence different types of damage. Hence, given the differences between Cr-reducing enzymes in rodents and humans, it is necessary to examine the types and levels of reactive intermediates produced by human enzymes.

We hypothesize: (1) that there are differences in some of the enzymatic mechanisms of Cr(VI) reduction in human liver vs. lung, and that lung microsomal enzymes can mediate Cr(VI) reduction at significant rates in the presence of O_2, and at Cr(VI) concentrations that are physiologically relevant; (2) that human Cr(VI)-reducing enzymes generate reactive intermediates, and that different enzymes generate different levels or types of intermediates; in the presence of O_2, ROS may be formed; (3) that iron will influence both the types and levels of these reactive intermediates; and (4) that the generation of these reactive intermediates can lead to various types of damage to cellular components including DNA and lipids. The original specific aims of this project were to:

1. Examine Cr(VI) reduction by human lung microsomes from several individuals to determine the general characteristics of Cr(VI) reduction. We will use microsomes from several individuals to determine the extent of interindividual variability, and to determine if Cr(VI) reduction parameters correlate with other known properties of these microsomes. The data will be compared to available data on liver microsomes.
2. Examine the Cr(VI)-reduction linked generation of reactive intermediates produced by human microsomal enzymes, in the presence and absence of O_2. We will use ESR to assess the formation of Cr(V), and an indirect ESR measurement for Cr(IV). To assess the role of specific enzymes, experiments will include the use of purified enzymes (e.g. FMO, P450 reductase, cytochrome b_5), alone or in combination. Experiments in the presence vs. absence of O_2 will be compared as we would expect ROS to be formed only in its presence; ESR with spin traps will be used to assess the formation of ROS.
3. Examine the effect of iron on the levels and types of reactive intermediates produced during Cr(VI) reduction by lung and liver enzymes. The ability of iron to change the levels or types of reactive intermediates formed during Cr(VI) reduction will be assessed, including the contributions of specific enzymes as described in aim 2. "Iron-free" conditions will be compared to those in which different levels of ferric compounds have been added.
4. Examine the potential of human microsomal enzymes to generate various types of damage during Cr(VI) reduction, and determine if specific types of damage are associated with the activity of certain enzymes or the production of certain reactive intermediates. The in vitro experiments described in aims *2* and *3* will be extended to determine which types of potential damage (DNA strand breaks, Cr-DNA adducts, and oxidative damage to DNA and lipids) are formed by various enzymes or other experimental manipulations.

OVERVIEW OF OBJECTIVES ACCOMPLISHED:

While this project was originally designated for three years of funding, budget cutbacks within AFOSR resulted in the premature termination of this project. Since only 8 months of funding were provided (1 APR 99 – 30 NOV 99; plus a no-cost extension until 28 FEB 99), all of the aims of this project were not accomplished. Nonetheless, significant progress was made on aims #1, #2, and #3 of this project.

Most of the results for aim #1 have been obtained. We have found very little interindividual variability in basic Cr(VI)-reducing properties of lung tissue among four individuals. As was the case for liver, human lung microsomal enzymes can mediate Cr(VI) reduction at significant rates using Cr(VI) concentrations well below those anticipated for occupational exposure, and Cr(VI) reduction still occurs at significant rates even under full room air.

Significant progress has also been made on aim #2. Numerous EPR studies using human liver and lung microsomes have shown that both tissues generate significant and sustained levels of Cr(V), a reactive intermediate which may directly mediate some of the toxicity. Since Cr(V) is known to mediate hydroxyl radical formation via a Fenton-like reaction, it seems likely that Cr(VI) reduction by these microsomal enzymes could also lead to toxicity due to reactive oxygen species [16,22,34,35]. Cr(V) is also known to bind to DNA [36], so it could likely lead to the eventual formation of stable Cr-DNA complexes. Cr(V) was detected under both anaerobic and aerobic conditions. An indirect EPR method also demonstrated the generation of Cr(IV), and Cr(III) was detected by EPR later in the time courses. Overall, the results suggest a stepwise one-electron reduction process, in which both reactive intermediates, Cr(V) and Cr(IV), are generated. Completion of previous work with purified recombinant enzymes indicates that flavin-containing monooxygenase (FMO) can only explain $\leq$10 % of microsomal Cr(VI) reduction. The cooperative role of cytochrome b_5 with either NADPH:P450 reductase or NADH:b_5 reductase likely explains the vast majority of Cr(VI) reduction.

Progress has also been made towards aim #3. Previous results have shown that the presence of small amounts of iron can lead to marked increases in the rate of Cr(VI) reduction [25], and we have now shown that these rate changes lead to a more rapid generation and subsequent reduction of Cr(V), suggesting that Fe(II) can reduce both Cr(VI) and Cr(V). While not part of the specific aims, an analogous redox cycling effect with certain quinones was observed, with the semiquinone radicals stimulating the reduction of both Cr(VI) and Cr(V). Given the ubiquitous nature of quinones, this could have profound toxicologic consequences as the reactive chromium intermediates are generated much more rapidly in the presence of quinones or iron.

The significant progress in all of these areas has greatly extended our understanding of the mechanisms and consequences of microsomal Cr(VI) reduction, and has identified simultaneous exposure to chromium and quinones or iron as additional risk factors with potentially serious toxicologic consequences.

ACCOMPLISHMENTS, NEW FINDINGS, AND THEIR IMPLICATIONS:

Aim #1 Accomplishments:

The characterization of the kinetics and general parameters of microsomal Cr(VI) reduction using lungs from 4 individuals has largely been completed. Cr(VI) reduction by lung microsomes occurs at significant rates, and is only partially inhibited by atmospheric levels of O_2. The kinetics are very similar among all 4 individuals, with K_m values of 3.98 – 4.94 μM and V_{max} values of 2.36 – 3.60 nmol/min/mg (Table 1). The average lung K_m is 3.3-fold greater than that in human liver, but still well below expected occupational exposures. Of special note is that the K_m values for Cr(VI) in human lung and liver are 250- to 1600-fold lower, respectively, than the K_m in rat liver (Table 1). The average V_{max} in lung is 28% of that in human liver, but still is consistent with significant rates of Cr(VI) reduction.

In contrast to the extreme O_2 sensitivity of microsomal Cr(VI) reduction in the rat, atmospheric O_2 inhibited only a minority of the human Cr(VI) reduction in both liver and lung (Table 1), suggesting that these enzymes can function at significant rates even in tissues with high O_2 tensions. Comparable to human liver, the general flavoprotein inhibitor $TlCl_3$ caused near complete inhibition of lung microsomal Cr(VI) reduction (Table 1), implicating a strong role for flavoproteins. Similar to liver, 100% carbon monoxide had no effect on Cr(VI) reduction by human lung (Table 1), implying that cytochromes P450 are not involved in either organ.

Overall Cr(VI) reduction rates in the lung are about one-fourth of those in the liver. The levels of P450 reductase and cytochrome b_5 in the lung are similarly about one-fourth of those in the liver (Table 1). These enzymes likely act together in both liver and lung as major contributors to Cr(VI) reduction.

For the one lung tested, *n*-octylamine (an FMO inhibitor) caused a 51.6% inhibition of Cr(VI) reduction. While *n*-octylamine caused a 19–28% inhibition of Cr(VI) reduction by liver microsomes, we have shown that FMO3, the major liver FMO isoform, has only limited capacity to reduce Cr(VI), i.e. it accounts for ≤10% of microsomal Cr(VI) reduction. However, the lung has different FMO isoforms so it is possible that FMO in the lung may have a role in Cr(VI) reduction.

In summary, there is little interindividual variability in basic Cr(VI)-reducing properties of lung tissue, and Cr(VI) reduction occurs at significant rates at Cr(VI) concentrations well below those anticipated for occupational exposure. Similar to the liver, microsomal flavoenzymes (e.g. P450 reductase, b_5 reductase) and cytochrome b_5 may cooperate to mediate Cr(VI) reduction in the lung.

Methods Used to Accomplish Aim #1:

Human Tissue. Human liver and lung tissues were provided by the Organ Transplant Unit at Froedtert Memorial Lutheran Hospital (Milwaukee, WI), with the approval of the Human Research Review Committee of the Medical College of Wisconsin. In all cases, the organs were removed from brain-dead organ transplant donors by the Organ Transplant Unit within approximately 30 min after death. The tissue was immediately iced, cut into small pieces, flash frozen in liquid nitrogen, and stored at –80°C as previously described [31]. A partial characterization of the P450 isozyme contents of the hepatic microsomes from four of these subjects has been published [6,42]. Microsomal fractions were prepared from human tissue using a conventional differential centrifugation procedure as previously described [31].

Cr(VI) reduction assay. Experiments to assess Cr(VI) reductase activity were conducted as previously described [31], using an NADPH-regenerating system (3 mM $MgCl_2$, 1 mM NADP, 7 mM G6P, and 0.4 U G6P dehydrogenase ml^{-1}) at 37°C. Microsomes were pre-incubated for 5 min prior to the addition of Na_2CrO_4 to a final concentration of 19.6 μM. Net enzymatic rates were obtained by subtracting the slow rates (the result of a slow chemical reduction by NADPH [19]) that were observed in the presence of pre-boiled microsomes (which were previously shown to lack enzymatic activity [31]). The reduction of Cr(VI) was stopped by the addition of 125 μl 2 M Na_2CO_3 (per 2.5 ml reaction volume) [19]. The concentration of remaining Cr(VI) was measured colorimetrically by 1,5-diphenyl-carbazide (DPC) in acid solution (pH 2) [33] after removal of

interfering reducing material by a charcoal/aluminum oxide mixture [32]. Cr(VI) concentrations were determined from a standard curve with Na_2CrO_4 as the standard by measuring absorbance at 540 nm against a blank in which Na_2CrO_4 was omitted. In some experiments, the NADPH-dependence of the reduction of Cr(VI) was assessed by removing individual components of the NADPH-generating system.

Experiments to examine the kinetics of microsomal Cr(VI) reduction were performed in an analogous manner, except that different concentrations of Na_2CrO_4 were used and all reactions were stopped after 3 min.

Experiments under anaerobic conditions (4 to 5% H_2/balance N_2) were conducted in a Coy anaerobic chamber (Coy Laboratory Products, Inc., Grass Lake, MI). All buffers, etc., were pre-incubated in the anaerobic chamber for at least one hour before use. Cr(VI) reduction rates under these conditions were found to be indistinguishable from those in which the vials were made anaerobic by flushing with O_2-free N_2. For those experiments conducted under aerobic conditions, open-top vials were incubated in a shaking waterbath (100 rpm) during all steps; for these vials, the reagents were not pre-incubated under anaerobic conditions.

Effects of Potential Inhibitors on Cr(VI) Reduction. The effect of CO on Cr(VI) reduction was examined as follows: microsomal preparations that had been rendered anaerobic were gassed with 100% CO on ice for 2 min and then pre-incubated for 5 min at 37°C prior to addition of Na_2CrO_4 in the Cr(VI) reduction assay. Controls were gassed under identical conditions with O_2-free N_2, which was generated by passing 100% high purity dry grade N_2 through an Oxi-Clear cartridge (Labclear, Oakland, CA).

For the other inhibitor experiments, the inhibitor was added at the same time as the NADPH-generating system and was therefore exposed to the microsomes during the 5 min pre-incubation period. The inhibitor stock solutions were made just before use and utilized as described [25]. For each inhibitor, Cr(VI) reduction rates were compared to those in the presence of the inhibitor solvent only. Pretreatment of microsomes with 0.2 mM 2-bromo-4′-nitroacetophenone (BNAP), which inhibits microsomal NADPH:cytochrome *c* reductase, was done as described [12], and compared against controls in which BNAP was omitted.

Miscellaneous Procedures. Microsomal protein concentrations were determined by the Lowry method [18] modified as described to remove potential interference from detergents [7], with bovine serum albumin as the standard. Total cytochrome P450 content was determined from CO-reduced minus reduced spectra as described [29] using an Aminco DW-2000 spectrophotometer (SLM-Aminco Instruments, Urbana, IL); the difference in absorbances at 450 and 490 nm was used to calculate the molar concentration of P450, using an extinction coefficient of 91 mM^{-1} cm^{-1} [9].

Determination of Enzyme Activities. FMO3 activity was assessed as oxidation of methyl *p*-tolyl sulfide using a method adapted from Brunelle et al. [4]. Specifically, the reaction was conducted in 50 mM potassium phosphate (pH 8.4) containing an NADPH-generating system (0.5 mM NADP, 2.0 mM G6P, 2 U G6P dehydrogenase ml^{-1}), 0.8 mM DETAPAC, 1 μM FAD. The FMO3 source was added and incubated with slow shaking for 2 min at 37°C. Methyl *p*-tolyl sulfide was added to a final concentration of 0.5 mM, and incubation was for 40 min at 37°C. Final total reaction volume was 0.25 ml. The reaction was stopped by adding 0.70 ml ice-cold acetonitrile, after which the tube was briefly vortexed and immediately iced. 20 mg NaCl was added to each tube, after which the tube was extensively vortexed. Following a 10 min centrifugation at 2000 x g at 4°C to separate the phases, the organic layer was analyzed by isocratic HPLC (mobile phase: 50% acetonitrile in water, 1.0 ml/min; Alltech C8 RSIL column, 250 x 4.6 mm; UV detection at 220 nm). The substrate (methyl *p*-tolyl sulfide) and product (methyl *p*-tolyl sulfoxide) were quantified by comparison of peak areas relative to those of standards of these two compounds.

NADH:cytochrome b_5 reductase activity was determined as NADH-dependent ferricyanide reduction as described [40,44]. NADPH:cytochrome P450 reductase activity was measured at 37°C as cytochrome *c* reduction [41]; the change in absorbance at 550 nm was followed over time in a 1-ml volume containing 0.1 μmol NADPH, 34 nmol cytochrome *c* "type III", 50 mM

potassium phosphate (pH 7.7), 1 mM cyanide, and 0.1 to 0.5 mg microsomal protein (ε = 29.5 mM^{-1} cm^{-1} for reduced cytochrome *c*).

Aim #2 Accomplishments:

Continued Development of Purified Enzymes Mediating Cr(VI) Reduction. Upon comparison of NADPH vs. NADH as electron donors, neither of which contained detectable levels of iron, the rate of microsomal Cr(VI) reduction with NADH was approximately twice that with NADPH (Fig. 1); both electron donors can therefore support significant microsomal Cr(VI) reduction. If these donors supplied independent pathways, then the rate with NADH+NADPH should be the sum of the individual rates. However, the rate with NADH+NADPH was not significantly greater than that with NADH alone (Fig. 1), suggesting that both electron donors are feeding electrons through a common component. The most likely component is cytochrome b_5, as it can be reduced by NADPH:P450 reductase and even more efficiently by NADH:b_5 reductase [8]. In support of this, when purified recombinant cytochrome b_5 was added to human N microsomes, the rate of Cr(VI) reduction more than doubled [25]. In addition, NADH-dependent Cr(VI) reduction by liver microsomes was completely inhibited by *p*-hydroxymercuribenzoate, a b_5 reductase inhibitor. The P450 reductase inhibitors 2-bromo-4′-nitroacetophenone [12] and diphenyliodonium chloride (an irreversible inhibitor of P450 reductase) [2] inhibited 80–85% and 100%, respectively, of NADPH-dependent human microsomal Cr(VI) reduction.

Purified recombinant human cytochrome b_5 and P450 reductase are available from PanVera Corp. (Madison, WI). These recombinant proteins have been used as antigens in rabbits to generate polyclonal antibodies (IgGs). The antibodies are specific for their respective antigens in Western blots of human microsomes, and the IgG specific for P450 reductase significantly inhibited its marker activity. To verify the interactions of these purified enzymes in reconstituted liposomes, P450 reductase mediated the NADPH-dependent reduction of cytochrome b_5 (Fig. 2A) as expected, whereas b_5 was not reduced directly by NADPH (Fig. 2B). While the ratios still need to be optimized, the interaction of P450 reductase and cytochrome b_5 in mediating iron(III) reduction is clear (Table 2).

Generation of Cr(V). We have conducted numerous experiments to determine the generation of Cr(V), a reactive intermediate, during Cr(VI) reduction by human microsomal enzymes. EPR spectra collected at 77 K clearly showed the production of a prominent Cr(V) signal (g = 1.981) during the reduction of Cr(VI) by human microsomes (Fig. 3a); close inspection of the spectra revealed asymmetry and fine structure in the g = 1.981 line, possibly suggesting multiple Cr(V) complexes. The Cr(V) signals were very small when using pre-boiled microsomes (Fig. 3b) or when NADP was excluded from the NADPH-generating system (Fig. 3c). These results are consistent with an active role for NADPH-dependent microsomal enzymes in generating most of the Cr(V). Since Cr(V) is potentially reactive and therefore unstable, the signal intensities do not represent absolute amounts of Cr(V) produced, but rather relative levels at that point in time.

Various time courses examined the Cr(V) signal intensity relative to the disappearance of Cr(VI). As an example, using hepatic microsomes under anaerobic conditions, the rate of Cr(VI) reduction was approximately linear over time, and a prominent Cr(V) signal was detected at the earliest time point (Fig. 4). Later, the Cr(V) signal diminished at a rate that paralleled that of Cr(VI) reduction, and the Cr(V) signal disappeared when all of the Cr(VI) had been reduced (Fig. 4). As shown (Fig. 4), human microsomal Cr(VI) reduction can result in the presence of Cr(V) over a long period of time. Cr(V) was also detected under aerobic conditions, but the signal was smaller, consistent with the slower rate of Cr(VI) reduction in the presence of O_2. Since Cr(V) does not continually accumulate over time, Cr(V) appears as a transient intermediate in a stepwise reduction of Cr(VI).

Generation of Cr(IV). Because Cr(IV) is highly reactive, it is difficult to detect directly; its formation has been inferred from indirect EPR measurements which are based on: Mn(II) + Cr(IV) → Cr(III) + Mn (III) [36,37]; this reaction implies that the loss of Mn(II) should correspond to the cumulative amount of Cr(IV) that has been generated up to that point in time. To verify detection of Mn(II), we obtained the expected prominent 6-line Mn(II) EPR signal (g = 1.995 and $A_{^{55}Mn}$ =

95.6 G) (Fig. 5 top); the height of the Mn(II) signal correlated strongly (linear correlation coefficient, $r = 0.982$) with variations in the concentration of Mn(II) over the range examined (25–400 μM). In a preliminary experiment with human microsomes, $MnCl_2$ was included at 400 μM, equal to that of Cr(VI), and the loss of the Mn(II) signal inferred the presence of Cr(IV) (Fig. 5, bottom); the loss of Mn(II) lagged behind, but was roughly parallel to, the loss of Cr(VI). This approach does not necessarily provide absolute quantitation [37], but at least suggests the formation of Cr(IV), the onset of which lags behind that of initial Cr(V) appearance.

Generation of Cr(III). In preliminary studies, the broad ESR signal (g = 2.02–2.03, line width of ~700–800 G) for Cr(III) [39] was detected later in microsomal time courses, after the disappearance of Cr(VI) and Cr(V) (not shown). The overall results are consistent with a consecutive single-electron stepwise reduction of Cr(VI) → Cr(V) → Cr(IV) → Cr(III).

Methods Used to Accomplish Aim #2

EPR Parameters. Cr(V), a d^1 paramagnetic species, has a distinct EPR spectrum at conventional X-band frequency that consists of a sharp line at g = 1·98 [22,37]. Relative changes in the levels of Cr(V) over time were estimated by comparison of changes in the signal intensity [37]. Cr(VI) reduction experiments were established as described above except that initial Cr(VI) levels were 400 μmol l^{-1} to provide an adequate signal for detection by EPR. Aliquots (0·3 ml) of the Cr(VI) reduction assays were taken at periodic intervals and frozen in quartz tubes by immersion in liquid nitrogen (77 K). The samples were stored at 77 K for up to one week until analyzed by EPR; we have demonstrated that Cr(V) is stable for at least several months at 77 K. The samples in the quartz tubes were placed in a finger Dewar with liquid nitrogen, and the EPR spectra were recorded using a Varian Century Series Spectrometer, which includes a Gauss meter for magnetic-field calibration and a frequency counter. Instrument settings for Cr(V) were as described in Fig. 3. For Cr(III), most settings were the same as for Cr(V), except for the following changes: 6 dB microwave power, a sweep width of 4000 G with a field set of 2000 G, and a 4 min scan time. Representative scans were repeated to verify reproducibility. The EPR parameters for Mn(II) were as described in Fig. 5.

Liposomes. Liposomes containing P450 reductase alone, cytochrome b_5 alone, and P450 reductase plus cytochrome b_5 were reconstituted as described [43], and consisted of phosphatidylcholine/phosphatidylethanolamine/ phosphatidylserine (ratio of 10/5/1, which approximates microsomal lipid composition [43]).

Reduction of Ferric Compounds by Microsomal Enzymes. Ferric reductase activity was assayed under anaerobic conditions by monitoring the production of Fe(II) over time as adapted from Myers and Myers [24]. All solutions were pre-incubated in the anaerobic chamber for at least one hour before use. The assays were performed at 37°C in temperature-controlled stirred anaerobic cuvettes using an Aminco anaerobic cell accessory (SLM Instruments, Urbana, IL); the assay mix (3.0 ml total volume) consisted of: 28 mM potassium phosphate buffer (pH 7.5), 0.4 mM ferrozine, 0.12 mM ferric-ADP, and microsomal enzymes as specified. The anaerobic cuvettes were set up in an anaerobic chamber in a 37°C heating block, and sealed before removal from the chamber to maintain anaerobic conditions. The assay was started by the addition of 60 μl 50 mM anaerobic NADPH to the sample cuvette (using the sealed plunger device of the anaerobic cell accessory); the reference cuvette received 60 μl anaerobic water at the same time. Activity was followed by an increase in absorbance over 3 min at 562 nm. Rates of Fe(III) reduction in the absence of enzymes, which were minimal, were subtracted from enzymatic rates to obtain the net values due to enzymatic activity. Using the extinction coefficient for the ferrozine-Fe(II) complex of 28 mM^{-1} cm^{-1} [5], changes in absorbance were converted to nmol of ferrozine-Fe(II) complex formed (equivalent to nmol Fe(III) reduced) per min at 37°C. In some experiments, other iron compounds were substituted for ferric-ADP.

Cr(VI) reduction assay. The details are the same as described for aim #1.

Aim #3 Accomplishments

Effects of Iron. We previously reported that trace amounts of iron could dramatically stimulate microsomal Cr(VI) reduction [25]. Because Fe(II) can directly reduce Cr(VI) [21,30], it seems that a small amount of Fe is repeatedly redox-cycled, resulting in the reduction of a much larger amount of Cr(VI). This has potentially significant toxicologic consequences, as the rate and extent of formation of reactive Cr intermediates could be greatly amplified by relatively small increases in intracellular iron. It was not clear, however, if human microsomal Cr(VI) reduction is totally linked to iron, or if there is some portion independent of iron. Extrapolation of the previous iron-dependent rate data back to zero iron implied that ~10–30% of the maximal Cr(VI)-reducing activity is 'iron-independent' [25]. To approach this from another perspective, we included deferroxamine, a strong Fe(III) chelator, in some experiments; the resulting data suggest an 'iron-independent' basal rate of Cr(VI) reduction that is 29–31% of that achieved with high iron (Fig. 6).

Iron not only affects the rate of Cr(VI) reduction [25], but it also influences the duration over which the Cr(V) signal is seen. For example, when using liver microsomes and NADH as the electron donor, a prominent Cr(V) signal persisted over a 60-min time course (Fig. 7A) because all Cr(VI) had not yet been reduced. However, when 12.4 μM $FeCl_3$ was included, Cr(VI) was essentially completely reduced by 50 min, at which time the Cr(V) signal also disappeared (Fig. 7B). Therefore, even though Cr(V) was generated more quickly in the presence of iron, the signal intensity was not significantly greater and the Cr(V) signal declined more quickly, and at a rate that roughly corresponded to the rate of Cr(VI) reduction (Fig. 7B). The overall results suggest that the redox cycling of a small amount of iron can facilitate the reduction of both Cr(VI) and Cr(V) according to the following reactions:

$$Fe(II) + Cr(VI) \rightarrow Fe(III) + Cr(V)$$
$$Fe(II) + Cr(V) \rightarrow Fe(III) + Cr(IV).$$

Iron had a similar effect on the rate of Cr(VI) [25] and Cr(V) reduction catalyzed by lung microsomes (not shown). While most EPR experiments were conducted at 400 μM Cr(VI) to enhance the detection of Cr(V) signals, in experiments using 100 μM Cr(VI), the Cr(V) signals were still readily detectable.

Effects of Quinones. While not an original aim or hypothesis of this project, a significant role for quinones in human microsomal Cr(VI) reduction was discovered. Because redox-active quinones are widespread (e.g. ubiquitous in all respiring plants and animals; automobile/diesel exhaust; cigarette smoke; urban air particulates; agricultural fungicides; etc. [23,28]), they represent another class of compounds that could significantly influence Cr(VI) reduction and Cr-related toxicity. Similar to the effects of iron, anaerobic Cr(VI) reduction by lung (not shown) and hepatic microsomes (Fig. 8) was markedly stimulated by 1,4-naphthoquinone (NQ) or 2-methyl-1,4-naphthoquinone (MNQ) [27]. The most pronounced stimulation occurred when quinones were increased from 1 to 5 μM, well below the Cr(VI) concentration; it is therefore likely that NQ and MNQ are being redox-cycled through their semiquinone radicals, and that these radicals are reducing Cr(VI). Within the other classes of compounds, methyl viologen, azobenzene, and 2,4-dinitrophenylhydrazine caused only minimal stimulation [27].

Aerobic experiments using increasing initial concentrations of Cr(VI) did show a stimulatory effect for quinones, which varied with the quinone and with the ratio of O_2 to Cr(VI) (Fig. 9). Compared to the methanol control, 10 μM NQ stimulated aerobic Cr(VI) reduction by 1.7- to 2.3-fold over the range of 20-400 μM initial Cr(VI) (Fig. 9a). While this is less than the 2.8- to 3.9-fold stimulation by NQ under anaerobic conditions (Fig. 8), it still represents a significant stimulatory effect. This relative effect is not significantly affected by the relative proportions of Cr(VI) to O_2 (Fig. 9a). In contrast, 10 μM MNQ stimulated aerobic Cr(VI) reduction by 2.2-fold only when initial Cr(VI) was 400 μM (Fig. 9b). While this is a significant stimulation, it is clearly less than the 9.6-fold stimulation caused by MNQ under anaerobic conditions (Fig. 8). At lower initial Cr(VI) concentrations, however, the effect of MNQ was minor or nonexistent (Fig. 9b). The differences between NQ and MNQ under aerobic conditions are consistent with the fact that MNQ• (the semiquinone of MNQ) is much more reactive with O_2 than is NQ• [28]. It therefore seems

likely that, unless Cr(VI) is in excess relative to O_2, MNQ• is preferentially oxidized by O_2, and therefore not available for the reduction of Cr(VI) (see below).

These aerobic studies were extended to examine the effects of varied quinone concentrations while maintaining a constant initial Cr(VI) level of 100 μM. Under these conditions, O_2 concentration is at least twice that of initial Cr(VI). In this case, 10 μM NQ caused a 2.15-fold stimulation of Cr(VI) reduction, while 25 μM NQ caused a 3.5-fold stimulation (Fig. 10). MNQ concentrations of 10 μM or less had no effect, whereas 25 and 50 μM MNQ caused 2.3- and 3.6-fold stimulation of Cr(VI) reduction, respectively (Fig. 10). Thus, even when there is less Cr(VI) than O_2, MNQ can stimulate Cr(VI) reduction, but it requires higher MNQ concentrations.

The overall quinone effects in the lung were similar to those observed with hepatic microsomes, except that higher quinone concentrations were required to attain the maximal rates in lung.

To determine if the effects of NQ and MNQ on microsomal Cr(VI) reduction were NADPH-dependent, experiments were done in which individual components of the NADPH-generating system were omitted. For both NQ and MNQ, the absence of either NADP or G6P essentially abolished the Cr(VI) reduction rates (Table 3), indicating that their effects are likely mediated through the action of microsomal NADPH-dependent enzymes. In support of this, addition of increasing levels of MNQ or NQ caused prominent increases in Cr(VI) reduction by purified recombinant human P450 reductase (Fig. 11). However, P450 reductase alone accounts for only a small percentage of microsomal Cr(VI) reduction, including that stimulated by quinones, so it is likely acting cooperatively with other microsomal components (e.g. cytochrome b_5).

If NQ and MNQ are exerting their NADPH-dependent effects on microsomal Cr(VI) reduction by redox cycling through their semiquinone radicals, then they should stimulate NADPH oxidation by microsomes. Rates of NADPH oxidation by hepatic microsomes were examined aerobically in the presence of 10 μM amounts of the various organic compounds. Both NQ and MNQ markedly stimulated microsomal NADPH oxidation. For hepatic microsomes, NADPH oxidation was negligible in the absence of either quinone (Fig. 12). Addition of increasing levels of MNQ up to 5 μM caused corresponding increases in NADPH oxidation (Fig. 12). Further rate increases were observed with 15 μM MNQ, but the slope of the rate change between 5 and 15 μM MNQ was not as great as that between 0 and 5 μM MNQ (Fig. 12). The shape of the MNQ curve for NADPH oxidation (Fig. 12) is very similar to that for Cr(VI) reduction (Fig. 8). In a similar manner, addition of increasing levels of NQ resulted in increasing rates of NADPH oxidation by hepatic microsomes (Fig. 12). Further significant increases in NADPH oxidation rates were not seen above 5 μM NQ. These concentration-dependent effects of NQ on NADPH oxidation (Fig. 12) parallel those on Cr(VI) reduction (Fig. 8).

For both NQ and MNQ, the molar production of superoxide by hepatic microsomes was similar to that of NADPH oxidation (Fig. 12). This quinone-mediated production of superoxide under aerobic conditions supports the hypothesis that the semiquinone radicals are readily oxidized by O_2, which makes less semiquinone available for mediating Cr(VI) reduction under aerobic conditions (Figs. 9,10).

In analogous experiments with lung microsomes, both MNQ and NQ stimulated significant rates of NADPH oxidation (data not shown). The NADPH oxidation rates in the presence of 50 μM NQ or MNQ were inhibited 92–95% by diphenyliodonium chloride (data not shown), an irreversible inhibitor of NADPH:P450 reductase [2], suggesting a prominent role for this enzyme. In the presence of 25 μM NQ or MNQ under aerobic conditions, lung microsomes also generated superoxide at rates that were 1.3- to 2.0-fold those of NADPH oxidation (data not shown), consistent with the ability of O_2 to oxidize these semiquinones.

Since the rates of NADPH oxidation mediated by NQ and MNQ were significantly greater than the Cr(VI) reduction rates at each quinone concentration [27], the rate of reduction of NQ or MNQ is theoretically sufficient to support the observed stimulation in the rate of Cr(VI) reduction. This has significant toxicologic consequences, as the rate and/or extent of formation of reactive Cr intermediates could be greatly altered by relatively small intracellular increases in the concentration of redox-active quinones. Azo and nitroaromatic compounds, as well as methyl viologen (Paraquat), seemed to have little effect on Cr(VI) reduction rates [27].

Since the oxidation of semiquinone radicals by Cr(VI) would represent a one-electron process, it seems likely that Cr(V) would be the resulting immediate product, assuming that the quinone was limiting as in our experiments. Not only was Cr(V) detected, but the presence of NQ caused a more rapid disappearance of both Cr(V) and Cr(VI) (Fig. 13). Since the highly reactive intermediate Cr(IV) would likely be formed {Cr(V) + •Q → Cr(IV) + Q}, it is possible that extensive free radical damage could result, but this remains to be determined.

The extent of stimulation of Cr(VI) reduction mediated by NQ or MNQ was similar when using hepatic microsomes from two individuals. In previous studies comparing the rates and basic properties of human microsomal Cr(VI) reduction, there were no major interindividual differences among microsomes from five humans [25], suggesting that the effects of NQ and MNQ are likely to be similar in a large percentage of humans.

While both MNQ and NQ mediated significant increases in anaerobic microsomal Cr(VI) reduction rates, the stimulation by NQ was less prominent. Two primary factors could be responsible for this difference: the rate of reduction of the quinone to its semiquinone radical, or the relative ability of the semiquinone radical to mediate the reduction of Cr(VI). It would appear that the rate of reduction of the quinone is not responsible for these differences, because, based on its ability to stimulate NADPH oxidation, NQ is reduced much more quickly by microsomes. Therefore, it seems plausible that NQ causes less stimulation of Cr(VI) reduction than does MNQ because NQ• is less reactive with Cr(VI) than is MNQ•. Consistent with this is the fact that MNQ• is much more reactive with O_2 than is NQ• [28].

While the bulk of studies have implicated flavoproteins (e.g. NADPH:P450 reductase, NADH:b_5 reductase), acting alone or in concert with cytochrome b_5, as the primary mediators of the reduction of quinones to semiquinones [1,13-15,17], rat liver cytochrome P450 2B1 is capable of reducing doxorubicin to its semiquinone radical [11]. However, 100% carbon monoxide caused only a 8% inhibition of Cr(VI) reduction by human N hepatic microsomes in the presence of 10 μM MNQ (not shown), suggesting that human cytochrome P450s do not contribute significantly to the quinone-mediated stimulation of microsomal Cr(VI) reduction.

We examined the possible combined interactions of iron plus quinones and found that iron could mediate further increases in Cr(VI) reduction rates in addition to those seen in the presence of 10 μM NQ (Fig. 14). While this warrants further study, it suggests the possibility that the combination of iron plus quinones could further exacerbate Cr toxicity relative to the effects of either alone.

Methods Used to Accomplish Aim #3:

Determination of the Iron Content of Solutions. To determine the iron content of G6Ps and other solutions used in the chromium reduction assay, solutions were mixed with freshly prepared sodium hydrosulfite (final concentration, 10 mM) to reduce all iron to Fe(II). Different aliquots (50–500 μl) of these solutions were then processed through the ferrozine assay [26,38] to determine the concentration of Fe(II). Calculations were made to account for volume differences to determine the amount of Fe contributed by each solution. No iron was detected in the 10 mM sodium hydrosulfite solution.

Cr(VI) reduction assay and EPR spectroscopy. The details are the same as described for aims #1 and #2.

Effects of various organic compounds on Cr(VI) reduction. Stock solutions (2.5 mM) of each of the quinones were prepared in methanol just before use. Dilutions of these stock solutions were made in methanol, so that addition of 50 μl to a standard Cr(VI) reduction assay (2.5 ml total volume) resulted in the desired final concentration. These compounds were added to the Cr(VI) reduction assay 5 min prior to the microsomes (see above). For each compound, Cr(VI) reduction rates were compared to those in which an equal volume of methanol was included.

Quinone-mediated microsomal oxidation of NADPH. The assays were performed at 37°C in temperature-controlled cuvettes; the assay mix (1 ml total volume) consisted of: 0.3 M potassium phosphate buffer (pH 7.7), 0.1 mM EDTA, varied amounts of the quinone substrate (NQ or MNQ) and microsomal protein as indicated. The assay was started by the addition of

NADPH to a final concentration of 0.1 mM; a reference cuvette was set up in an identical manner except that an equal volume of methanol was substituted for the quinone substrate. The oxidation of NADPH was followed over time by a decrease in absorbance at 340 nm. No oxidation of NADPH was seen in the absence of microsomes. Using the extinction coefficient for NADPH of 6.22 per mM per cm [15], changes in absorbance were converted to nmol of NADPH oxidized per min at 37°C.

Production of superoxide during quinone reduction. The production of superoxide as a result of quinone (NQ or MNQ) reduction under aerobic conditions was performed at 37°C in temperature-controlled cuvettes [15]. The assay is based on the SOD-inhibitable reduction of acetylated cytochrome *c*. The assay mix (1 ml total volume) consisted of: 0.3 M potassium phosphate buffer (pH 7.7), 0.1 mM EDTA, varied amounts of the quinone substrate (NQ or MNQ) as indicated, 25 μM acetylated cytochrome *c*, and microsomal protein as indicated. The assay was started by the addition of NADPH to a final concentration of 0.1 mM; a reference cuvette was set up in an identical manner except that SOD was included at a final concentration of 667 U per ml [15]; this amount of SOD is needed to keep superoxide from reducing the quinones and ultimately cytochrome *c* [15]. The reduction of acetylated cytochrome *c* was followed over time by an increase in absorbance at 550 nm. Using the extinction coefficient 21.0 per mM per cm (which is the difference between extinction coefficients for the oxidized and reduced forms of cytochrome *c*) [3], changes in absorbance were converted to nmol of acetylated cytochrome *c* reduced per min at 37°C, which should be equivalent to nmol superoxide produced. Superoxide was not produced in the absence of microsomes, or in the presence of microsomes when an equal volume of the solvent methanol was substituted for NQ or MNQ.

NEW DISCOVERIES, CUMULATIVE:

1. There is little difference in chromium-reducing rates or properties when comparing lung microsomes from four humans.
2. The kinetic properties of chromium reduction are consistent with a potential significant role for *in vivo* Cr(VI) reduction, using Cr(VI) concentrations well below those anticipated for occupational exposure (apparent K_m for chromate of 1.04–1.68 μM in the liver, and 3.98–4.94 μM in the lung).
3. Relative to anaerobic conditions, Cr(VI) reduction by lung microsomes was inhibited only 16–34% by room air, suggesting that lung microsomal enzymes can still catalyze Cr(VI) reduction at significant rates even under the high O_2 tensions in lung.
4. Studies with lung microsomes from one human exhibited V_{max} and K_m values that were two-thirds lower and 2.8-fold greater, respectively, than those of hepatic microsomes from the same individual; other Cr(VI)-reducing parameters were similar for lung and liver.
5. Similar to human liver, lung microsomal Cr(VI) reduction is not catalyzed by cytochromes P450, but by flavoproteins. It is likely that NADPH:P450 reductase and NADH:b_5 reductase, in cooperation with cytochrome b_5, mediate the majority of Cr(VI) reduction by lung microsomes. Consistent with this, rates supported by NADH + NADPH are not greater than with NADH alone, suggesting that both electron donors mediate Cr(VI) reduction through a common intermediate, probably cytochrome b_5. Purified P450 reductase and cytochrome b_5 cooperate to mediate the reduction of iron(III).
6. During human microsomal Cr(VI) reduction, the reactive intermediate Cr(V) appears early and is generated over a long period of time. Cr(V) did not continually accumulate over time, but appears as a transient intermediate in a stepwise reduction of Cr(VI).
7. Using an indirect EPR method, the formation of Cr(IV) was inferred as a subsequent step in the continued reduction of Cr(V). The formation of this highly reactive intermediate could have significant toxicologic consequences.
8. Later in the time courses, Cr(III) was detected. The overall results are consistent with a consecutive single-electron stepwise reduction of Cr(VI) → Cr(V) → Cr(IV) → Cr(III).
9. Prior experiments on the link between Cr(VI) and Fe(III) reduction were extended:
   a. An 'iron-independent' basal rate of Cr(VI) reduction is 29–31% of that achieved with high iron.
   b. Iron not only affects the rate of Cr(VI) reduction by human liver and lung microsomes, but it also shortens the duration over which the Cr(V) signal is seen. So while Cr(V) is generated more quickly in the presence of iron, it is subsequently reduced more quickly as well, presumably to Cr(IV), a highly reactive species.
10. A significant role for quinones in human microsomal Cr(VI) reduction was discovered. Because redox-active quinones are widespread, they represent another class of compounds that could significantly influence Cr(VI) reduction and Cr-related toxicity:
    a. Similar to the effects of iron, anaerobic Cr(VI) reduction by lung and hepatic microsomes was markedly stimulated by small amounts of 1,4-naphthoquinone (NQ) or 2-methyl-1,4-naphthoquinone (MNQ). These quinones are being redox-cycled through their semiquinone radicals, and the semiquinones are reducing Cr(VI).
    b. Within other classes of compounds, methyl viologen, azobenzene, and 2,4-dinitrophenylhydrazine caused only minimal stimulation of Cr(VI) reduction.
    c. The stimulatory effect of the quinones under aerobic conditions were influenced by the quinone itself, the quinone concentration, and the ratio of O_2 to Cr(VI). It is clear that O_2 can compete with Cr(VI) for reaction with the semiquinones.
    d. The stimulation by the quinones is mediated by NAD(P)H-dependent microsomal enzymes.
    e. The addition of increasing levels of MNQ or NQ caused prominent increases in Cr(VI) reduction by purified recombinant human P450 reductase. However, P450 reductase alone accounts for only a small percentage of microsomal Cr(VI) reduction, including that

stimulated by quinones, so it is likely acting cooperatively with other microsomal components (e.g. cytochrome b_5).

f. Both NQ and MNQ markedly stimulate NADPH oxidation by microsomes. These concentration-dependent effects on NADPH oxidation parallel those on Cr(VI) reduction.
g. Under aerobic conditions, both NQ and MNQ stimulate the molar production of superoxide by hepatic microsomes to an extent similar to that of NADPH oxidation. This quinone-mediated production of superoxide under aerobic conditions supports the hypothesis that the semiquinone radicals are readily oxidized by O_2, which makes less semiquinone available for mediating Cr(VI) reduction under aerobic conditions.
h. The quinones caused a more rapid disappearance of both Cr(V) and Cr(VI). Since the highly reactive intermediate Cr(IV) would likely be formed, it is possible that extensive free radical damage could result.
i. There was little interindividual variability in the effects of the quinones, suggesting that their effects are likely to be similar in a large percentage of humans.
j. Human cytochrome P450s do not contribute significantly to the quinone-mediated stimulation of microsomal Cr(VI) reduction.

11. Iron can mediate further increases in Cr(VI) reduction rates in addition to those seen in the presence of NQ, suggesting the possibility that the combination of iron plus quinones could further exacerbate Cr toxicity relative to the effects of either alone.

LISTING OF COMMUNICATIONS AND INTERACTIONS:

Personnel Involved (1 APR 98 to 28 FEB 99):

Charles R. Myers, Ph.D., Principal Investigator. 30% effort, 1 APR 98 to 28 FEB 99.
William E. Antholine, Ph.D., co-Principal Investigator. 10% effort, 1 APR 98 to 30 NOV 98.
Judith M. Myers, M.S., Research Scientist. 20% effort, 1 APR 98 to 30 NOV 98.
Björn Porgilsson, B.S., Graduate Student. 20–100% effort, 1 APR 98 to 28 FEB 99 (stipend provided by MCW Dept. of Pharmacology & Toxicology).
Paul Jannetto, B.S., Graduate Student. 100% effort, 1 APR 98 to 28 FEB 99 (stipend provided by AASERT/AFOSR award F49620-97-1-0423 linked to this grant).
Brian P. Carstens, B.S., Research Associate, 90% effort: 1 APR 98 to 30 JUN 98; 95% effort: 1 JUL 98 to 30 NOV 98; 75% effort, 1 DEC 98 to 31 DEC 98.

Publications (1 APR 98 to 28 FEB 99):

C. R. Myers, B. Porgilsson, B. P. Carstens, and J. M. Myers. 1999. Naphthoquinones stimulate the rate of reduction of hexavalent chromium by human microsomes. *Toxic Substance Mechanisms* in press.

C. R. Myers, B. P. Carstens, and W. E. Antholine. 1999. Reduction of chromium(VI) to chromium(V) by human microsomes: effects of iron and quinones, in final preparation for submission to *Toxic Substance Mechanisms*.

The following was listed in the progress report for the previous 3-year period as 'in press' but it has since been published:

C. R. Myers and J. M. Myers. 1998. Iron stimulates the rate of reduction of hexavalent chromium by human microsomes. *Carcinogenesis* 19:1029–1038.

Meetings and Seminars (1 APR 98 to 28 FEB 99):

1. Seminar:
   12 MAR 1999, Biophysics Research Institute, Medical College of Wisconsin
   Title: "Reduction of Chromium(VI) by Human Microsomal Enzymes: Effects of Iron and Quinones on the Formation and Subsequent Reduction of Chromium(V)"

The seminar was attended by members of various departments including Biophysics, Pharmacology/Toxicology, Pathology, Biochemistry, and Cell Biology. The seminar resulted in a discussion of alternative approaches to assess the generation of reactive oxygen species as a result of chromium reduction by human enzymes.

Interactions (1 APR 98 to 28 FEB 99):

1. A collaboration was established with David Petering, Ph.D., Professor of Chemistry at the University of Wisconsin–Milwaukee, who is an expert in metal-binding proteins including metallothionein (MT). We conducted preliminary experiments comparing the ability of zinc-MT and apo-MT to reduce Cr(VI) relative to human microsomal enzymes. Zinc-MT was a poor mediator of Cr(VI) reduction, whereas apo-MT mediated Cr(VI) reduction at a moderate rate, although clearly less than that of microsomal enzymes. When present together, apo-MT and human microsomes mediated Cr(VI) reduction independent of the other. Since the in vivo role of MT is presumed to be protection against metal toxicity via metal chelation, we are planning to pursue a potential protective role for MT in quenching some of the reactive intermediates generated via human microsomal Cr(VI) reduction.

Consultative and Advisory Functions (1 APR 98 to 28 FEB 99):

My research efforts and results on this project directly led to the following opportunities to serve as an advisor, consultant, or collaborator on several other projects:

1. My development and use of antipeptide antibodies and their use in immunoblotting provided me with an opportunity to serve as a collaborator with Dr. N.C. Bols and co-workers (University of Waterloo, Ontario, Canada). This collaboration resulted in one publication during this period on the induction of cytochrome P4501A1 by PCBs and dioxin:
   J. H. Clemons, C. R. Myers, L. E. J. Lee, D. G. Dixon, and N. C. Bols. 1998. Induction of cytochrome P4501A by binary mixtures of polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in liver cell lines from rat and trout. *Aquatic Toxicology* 43:179–194.

2. My expertise, equipment, and studies on the potential role of cytochrome P450s in human chromium reduction for the AFOSR-funded studies provided me with an opportunity to serve as a collaborator with Dr. William Campbell (Medical College of Wisconsin) to elucidate the binding of nitric oxide to mitochondrial P450s, and the subsequent inhibition of aldosterone synthesis. This collaboration has resulted in one publication:
   C. J. Hanke, J. G. Drewett, C. R. Myers, and W. B. Campbell. 1998. Nitric oxide inhibits aldosterone synthesis by a guanylyl cyclase-independent effect. *Endocrinology* 139:4053–4060.

3. The collaboration on this project between myself and William Antholine, Ph.D., of the Biophysics Research Institute, established the EPR parameters necessary for studying the formation of chromium(V) by human microsomal enzymes. We were able to utilize these methods for another project on the reduction of chromium(VI) by an environmental bacterium; this process has potential bioremediation capabilities. This has resulted in the submission of one manuscript.
   C. R. Myers, B. P. Carstens, W. E. Antholine, and J. M. Myers. 1999. Chromium(VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1. Submitted to *Journal of Applied Microbiology*.

Technology Transfers (1 APR 98 to 28 FEB 99):

The following technology transfers are directly related to research tools derived from this project:

1. Customer: Dr. Adam Benham
   Biochemistry Department
   Academic Medical Centre
   Meibergdreef 15
   1105AZ Amsterdam
   Netherlands
   Telephone: 31 20 5665382
   FAX: 31 20 6915519

   Result: As one of the tools needed for our AFOSR project, we developed an antipeptide antibody that specifically recognizes the major human hepatic flavin-containing monooxygenase (FMO3). We described the development of this antibody in a publication (*J. Pharmacol. Toxicol. Meth.* 37:61–66; 1997). On 18 JAN 99, some of this antibody was transferred to Dr. Benham.

   Application: Dr. Benham is using the antibody for Western blots as a tool to detect FMO in experiments aimed at elucidating chaperones and protein folding pathways in the endoplasmic reticulum of hepatocytes.

Inventions (1 APR 98 to 28 FEB 99):

No inventions.

Honors/Awards (1 APR 98 to 28 FEB 99):

None.

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TABLES AND FIGURES

Table 1. Comparison of Cr(VI)-Reducing Properties of Rat vs. Human Microsomes

Property	Rat Liver	Human Liver	Human Lung
NAD(P)H-dependent	YES	YES	YES
K_m for chromate, μM	1250–1690[a]	1.04–1.68	3.98–4.94
V_{max} (nmol/min/mg)	6.3–6.5[b]	10.4–10.8[c]	2.36–3.60
% Inhibition of Cr(VI) reduction by:			
O_2, 21% (~210 μM)	n.t.[d]	26–37%	16–34%[e]
O_2, 1% (~10 μM)	100%[b]	n.t.	n.t.
O_2, 0.1% (~1 μM)	30%[b]	n.t.	n.t.
CO, 100%	28–50%[b]	none	none
metyrapone, 50 μM	27–51%[a]	none	n.t.
piperonyl butoxide, 0.12 mM	—[f]	none	n.t.
aminopyrine, 5.9 mM	—	none	n.t.
$TlCl_3$, 0.26 mM	—	96–100%	92–100%
Microsomal Content of:			
P450 reductase act. (nmol/min/mg)	—	73–139	26–48
Cytochrome b_5 (nmol/mg)	—	0.36–0.41	0.11–0.16[e]

[a] From Garcia & Wetterhahn-Jennette [10].

[b] From Mikalsen et al. [20].

[c] Earlier, the V_{max} for human N was reported as 5.03 nmol/min/mg [25], but this was obtained using a different lot of G6P for which the iron content was unknown (see below). In recent experiments using iron levels comparable to those used with the other individuals, human N had a V_{max} of 10.8 ± 1.05 nmol/min/mg.

[d] n.t., not tested.

[e] Results with all 4 lungs not yet available.

[f] —, not reported.

Table 2. NADPH-dependent iron(III) reduction in reconstituted liposomes with purified recombinant human cytochrome b_5 and P450 reductase

P450 reductase in liposomes	Cytochrome b_5 in liposomes	Fe(III) reduction* (nmol/min)
YES	no	0.0150
no	YES	none detected
YES	YES	0.0393

*Anaerobic reduction of Fe(III)-ADP was assayed as previously described [25].

Table 3. NADPH-dependence of quinone-mediated anaerobic Cr(VI) reduction by human liver microsomes[a]

Quinone[b]	NADPH-generating system	Cr(VI) reduction rate (nmol/min/mg)[c]
NQ	complete system	19.3 ± 0.8
NQ	minus NADP	0.7 ± 0.2
NQ	minus G6P	0.2 ± 0.2
MNQ	complete system	34.4 ± 0.0
MNQ	minus NADP	0.1 ± 0.2
MNQ	minus G6P	–1.1 ± 0.5

[a]Each assay included 0.26 mg human N microsomal protein.

[b]NQ and MNQ were present at 25 μM final concentration.

[c]Measured over a time course of 5 min; values represent the mean for $n = 2$, ± the range of high and low values.

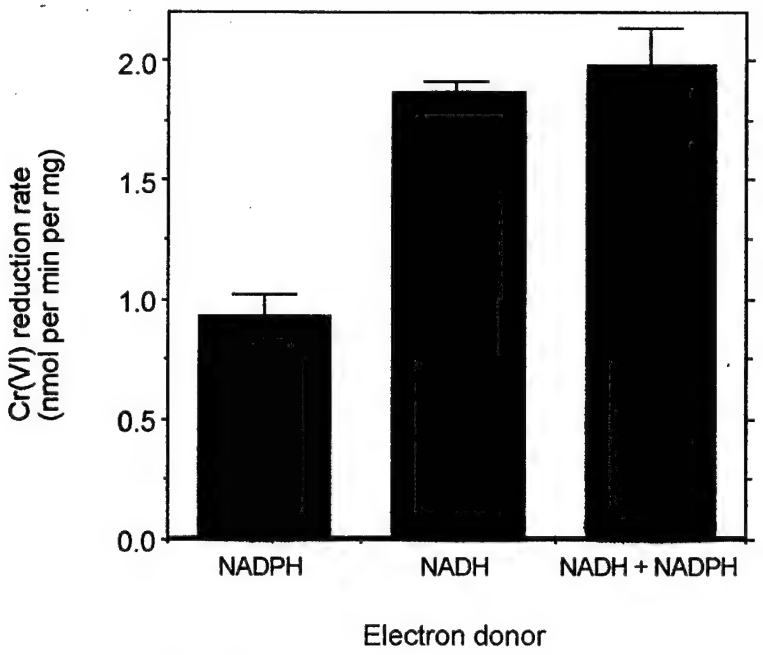

Fig. 1. Rates of Cr(VI) reduction by human N hepatic microsomes supported by NADPH, NADH, or NADH + NADPH (2 mM each). Results are mean ± S.D., $n = 3$.

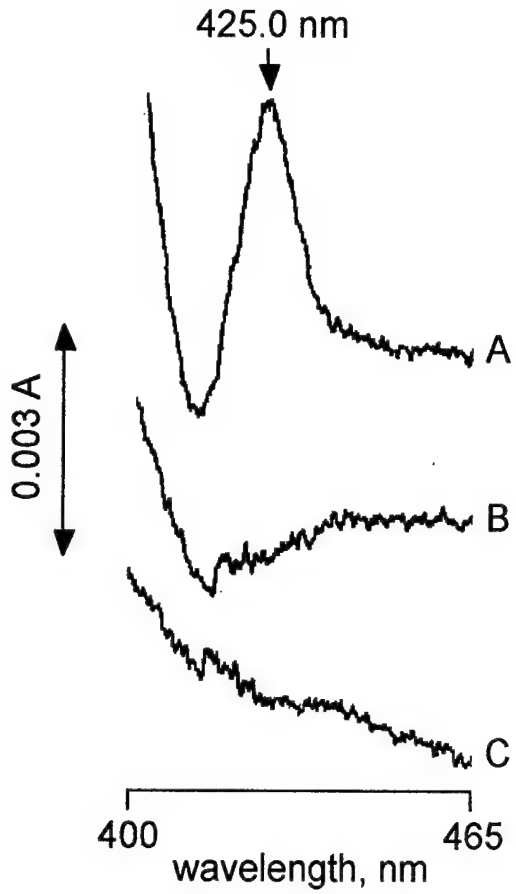

Fig. 2. NADPH-dependent reduction of cytochrome b_5 (Soret peak with maximum at 425 nm) in reconstituted liposomes containing 0.14 nmol each of b_5 and P450 reductase (**A**). No Soret peak was seen in liposomes containing b_5 alone (**B**) or P450 reductase alone (**C**). The liposomes were suspended in 2 ml 0.1 M phosphate buffer, pH 7.0, split between 2 cuvettes, and NADPH (final conc. 1 mM) was added to the sample cuvette. The peak in **A** appeared within 15 sec; the signal size did not change during repetitive scans over 10 min. Scans were corrected against a scan of NADPH in buffer, using the subtraction feature of the Aminco DW-2000 software. The scans were offset from each other vertically to facilitate visualization.

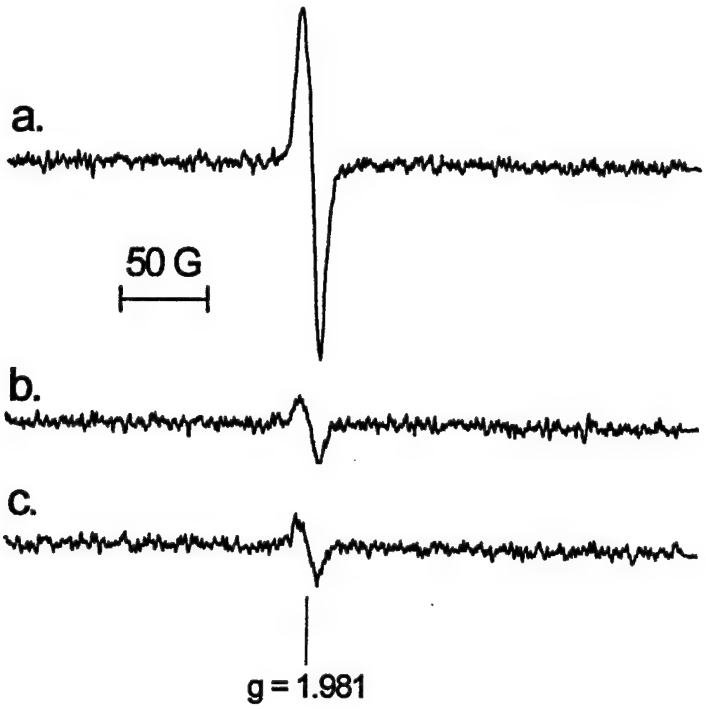

Fig 3. EPR spectra of Cr(V). For each experiment, 400 μM Na_2CrO_4 was added to our standard assay conditions [31] containing 1.56 mg human N hepatic microsomal protein, the NADPH-generating mix, and 6.5 μM $FeCl_3$. Incubation was for 5 min under anaerobic conditions, at which time they were frozen and stored in liquid N_2 (77 K) for EPR analysis. **a:** active microsomes plus complete system; **b**: pre-boiled microsomes plus complete system; **c**: same as **a** except that NADP was excluded. ESR instrument settings were: 1.0 G modulation amplitude, 4 x 10^4 receiver gain, 0.128 s time constant, 9.107 GHz microwave frequency, 34 dB microwave power, sweep width = 400 G, field set = 3300 G, modulation frequency = 100 kHz , scan time = 2 min.

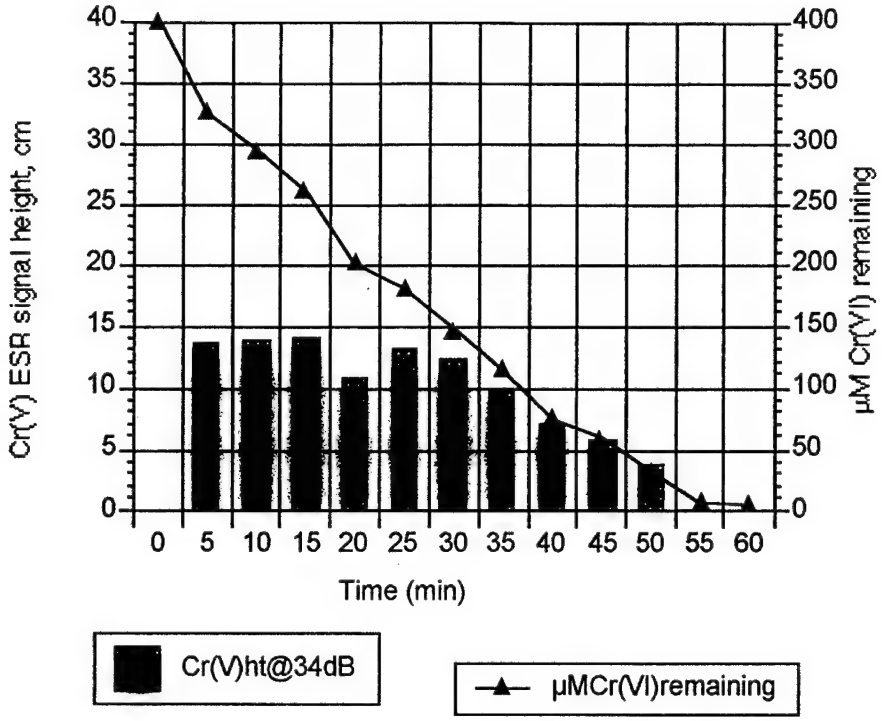

Fig. 4. Time course of Cr(V) EPR signal intensity at 34 dB (vertical bars) relative to the level of Cr(VI) remaining at each time point (▲). Conditions incl. 400 µM initial Cr(VI), human N hepatic microsomes (1.56 mg), and an NADPH-generating system. Samples were analyzed at 5-min intervals over the 60-min time course. A portion of each sample was frozen (77 K) for EPR analysis, while the rest was processed for Cr(VI) analysis. EPR instrument settings were the same as those for Fig. 3.

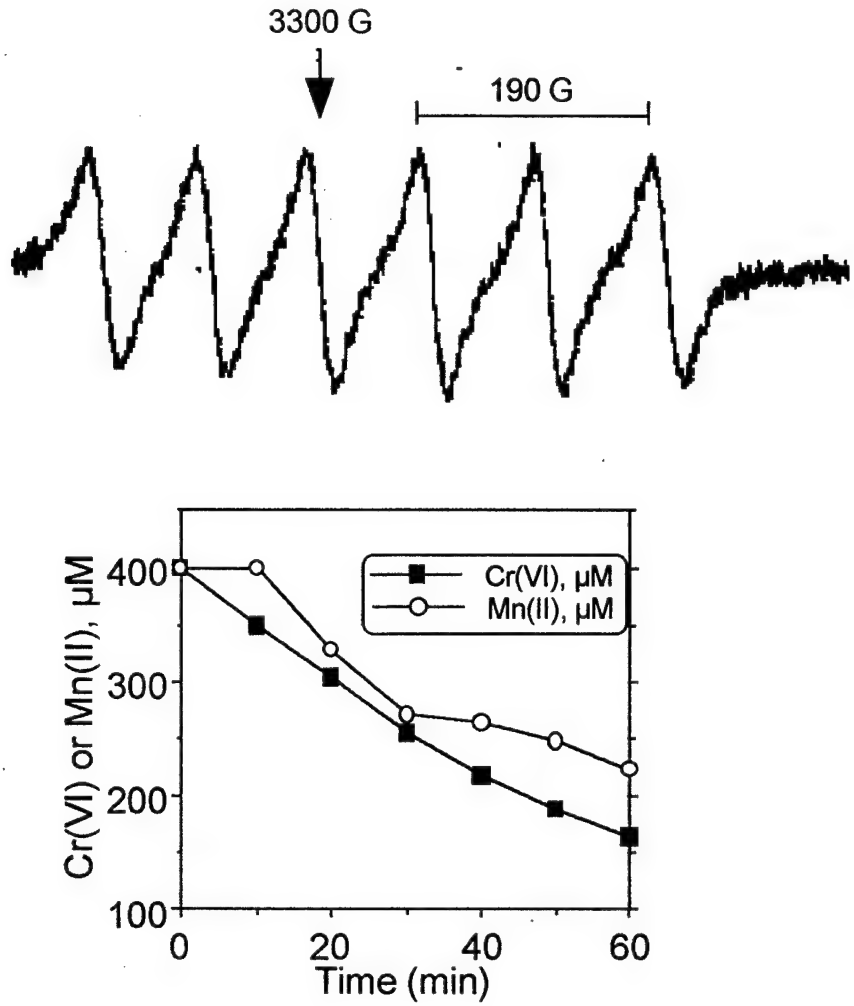

Fig. 5.
TOP: EPR spectrum of 200 μM Mn(II) in water. EPR instrument settings: 1.0 G modulation amplitude, 1 x 10^4 receiver gain, 0.016 s time constant, 9.490 GHz microwave frequency, 10 dB microwave power, sweep width = 1000 G, field set = 3300 G, 100 kHz modulation frequency, 1 min scan time.
BOTTOM: Loss of Mn(II) over time implies Cr(IV) formation as a consequence of NADPH-dependent Cr(VI) reduction by human hepatic microsomes.

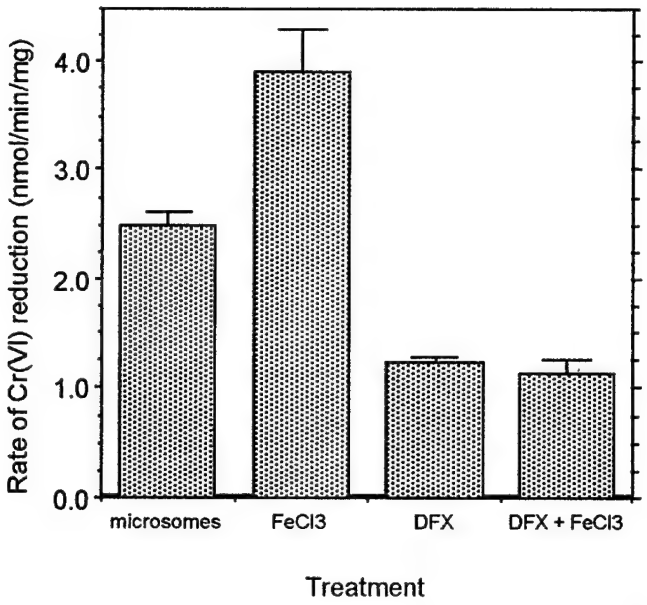

Fig. 6. Effect of 0.1 mM deferroxamine (DFX) on anaerobic Cr(VI) reduction by human N hepatic microsomes. The effect of DFX in the absence of added Fe represents its ability to chelate the iron contaminant in the G6P of the NADPH-generating system. 'FeCl3' includes 6.5 μM $FeCl_3$. Results = mean ± S.D., $n = 3$.

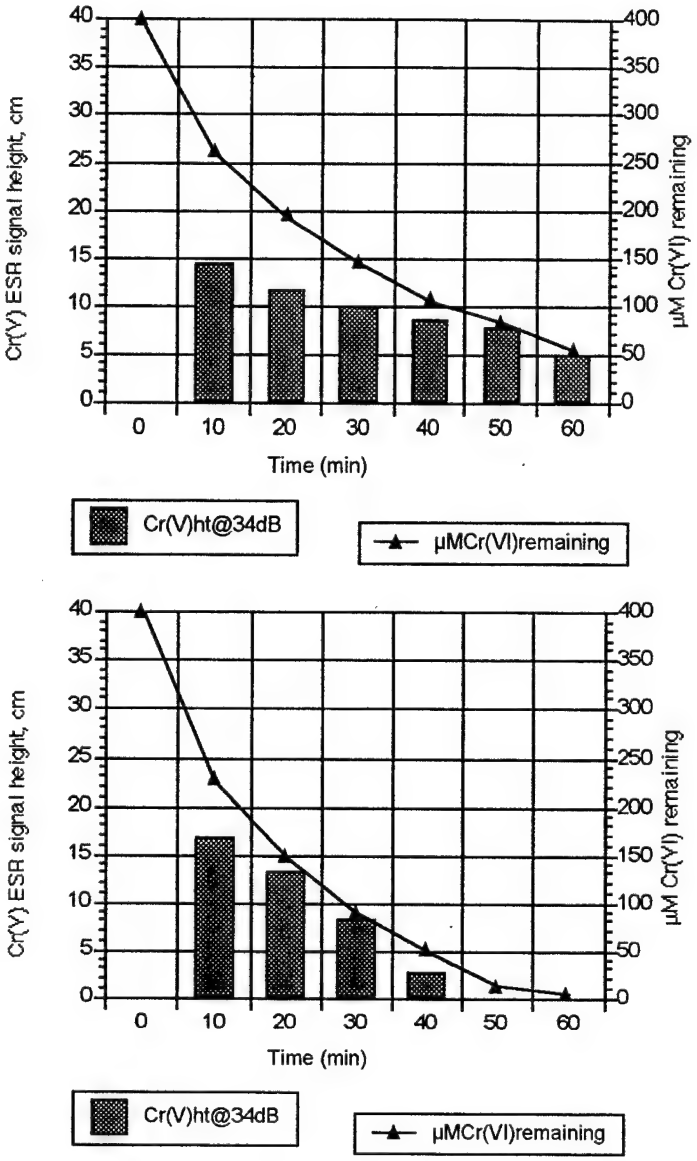

Fig. 7. Anaerobic time course of Cr(V) EPR signal intensity at 34 dB (vertical bars) relative to the level of Cr(VI) remaining (▲), using human N hepatic microsomes (1.56 mg), 400 µM initial Cr(VI), and 2 mM NADH.
TOP: active microsomes plus NADH;
BOTTOM: same as TOP but incl. 12.4 µM $FeCl_3$.
Samples were analyzed at 10-min intervals over the 60-min time course. A portion of each sample was frozen (77 K) for ESR analysis, while the rest was processed for Cr(VI) analysis. EPR instrument settings were the same as Fig. 3.

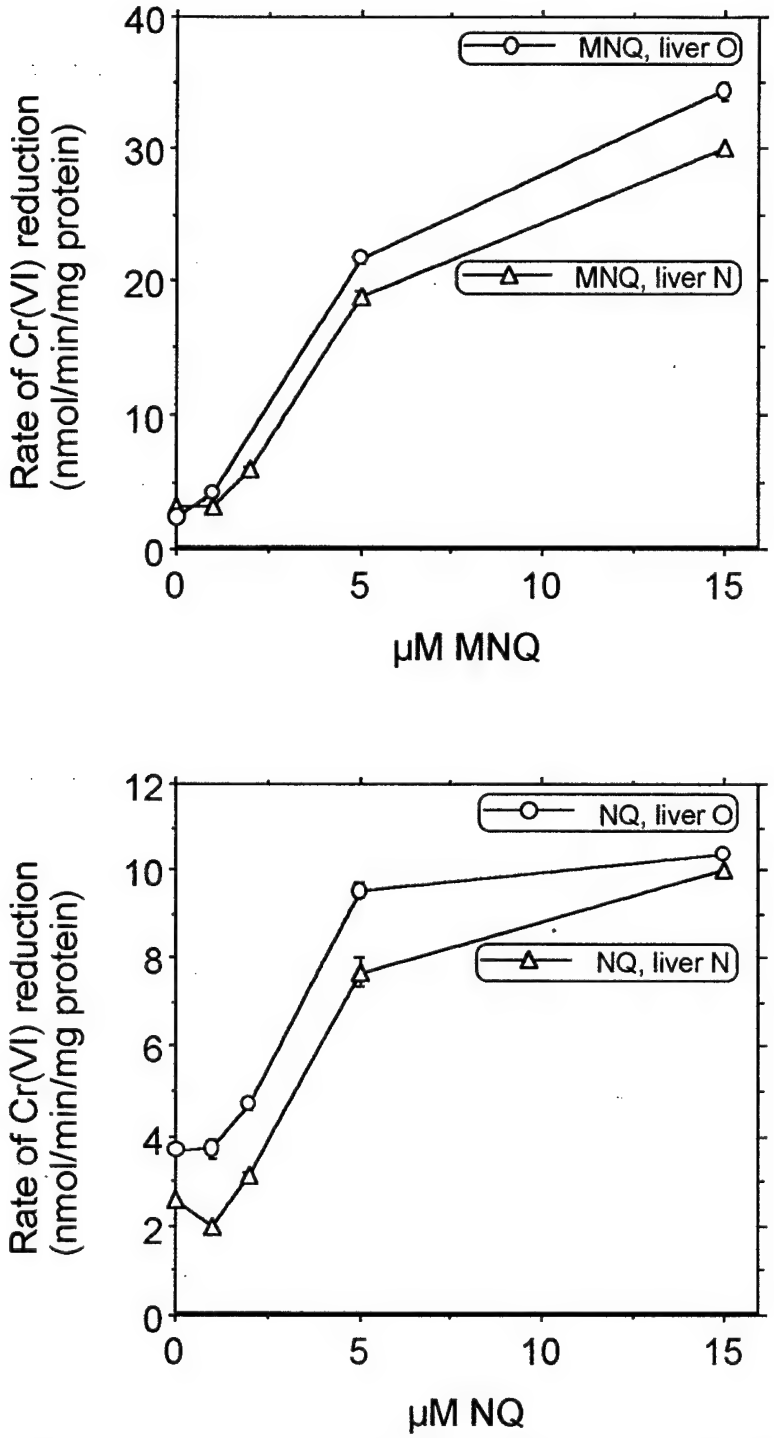

Fig. 8. Effects of adding increasing amounts of MNQ (**top**) or NQ (**bottom**) on anaerobic NADPH-dependent Cr(VI) reduction rates catalyzed by hepatic microsomes from human N (0.26 mg) or human O (0.26 mg). Initial Cr(VI) was 20 μM. Points represent the mean for $n = 2$, and bars represent the range of high and low values.

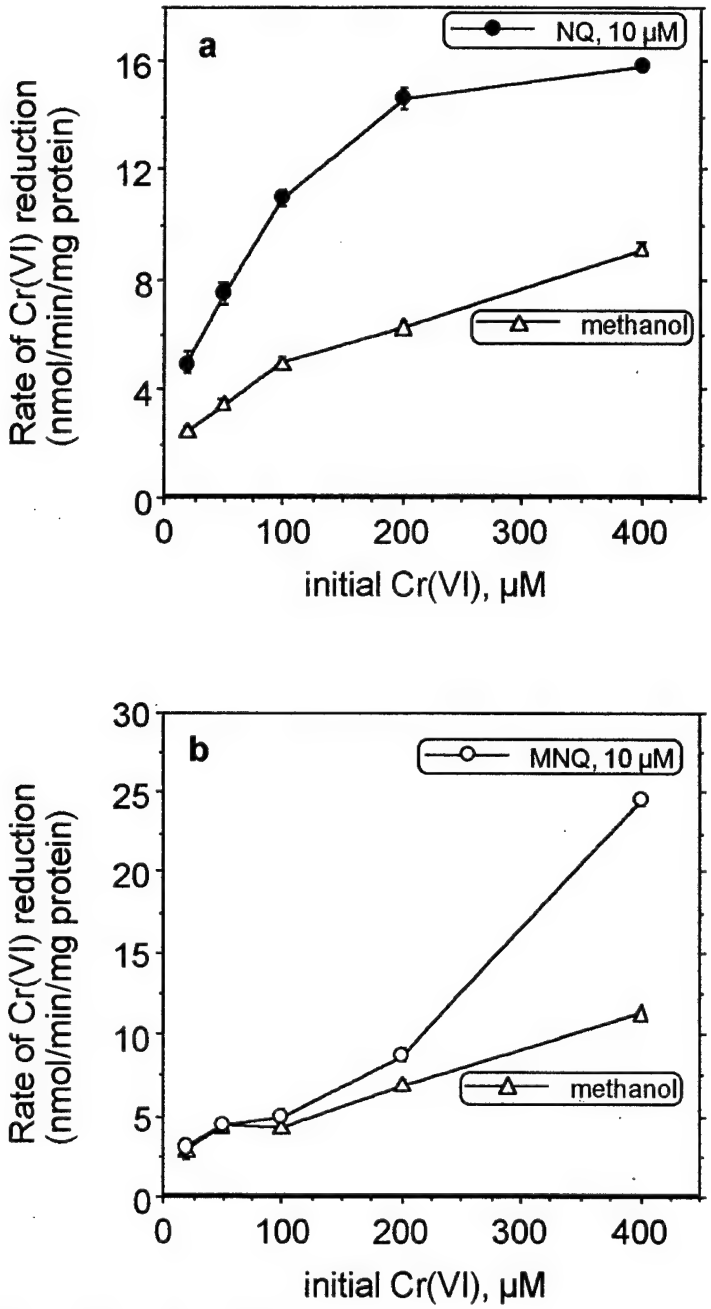

Fig. 9. Effect of adding 10 μM NQ (**a**) or 10 μM MNQ (**b**) on aerobic NADPH-dependent Cr(VI) reduction rates catalyzed by hepatic microsomes from human N. The rates are compared to those in which an equal volume of methanol (the quinone solvent) was substituted for the quinones. Each rate was determined over a 20–30 min time course, and initial Cr(VI) varied from 20–400 μM. Each experiment was conducted with a constant ratio of 0.378 mg of microsomal protein per 100 μM initial Cr(VI). Results shown represent the mean ± S.D., $n = 3$; for points lacking apparent error bars, the bars were smaller than the points as shown.

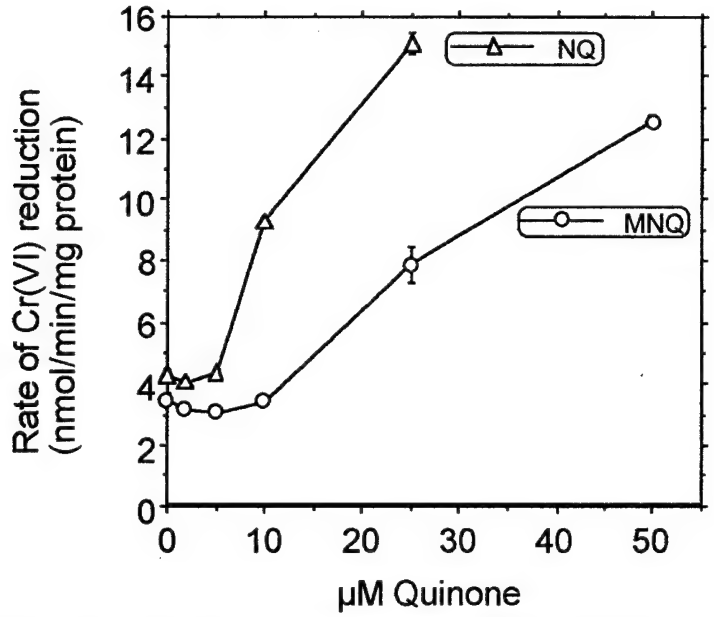

Fig. 10. Effects of adding increasing amounts of NQ or MNQ on aerobic NADPH-dependent Cr(VI) reduction rates catalyzed by hepatic microsomes from human N (0.378 mg). Each rate was determined over a 20–30 min time course, and initial Cr(VI) was 100 μM. Points represent the mean for $n = 2$, and bars represent the range of high and low values; for points lacking apparent range bars, the bars were smaller than the points as shown.

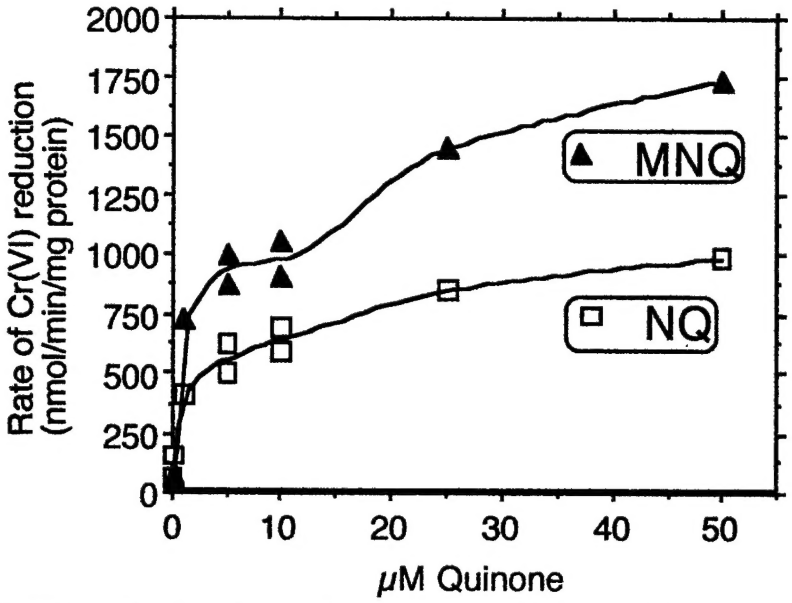

Fig. 11. Effect of adding increasing amounts of NQ or MNQ on anaerobic Cr(VI) reduction rates catalyzed by purified recombinant human P450 reductase (2 μg). Data points from two independent experiments are included.

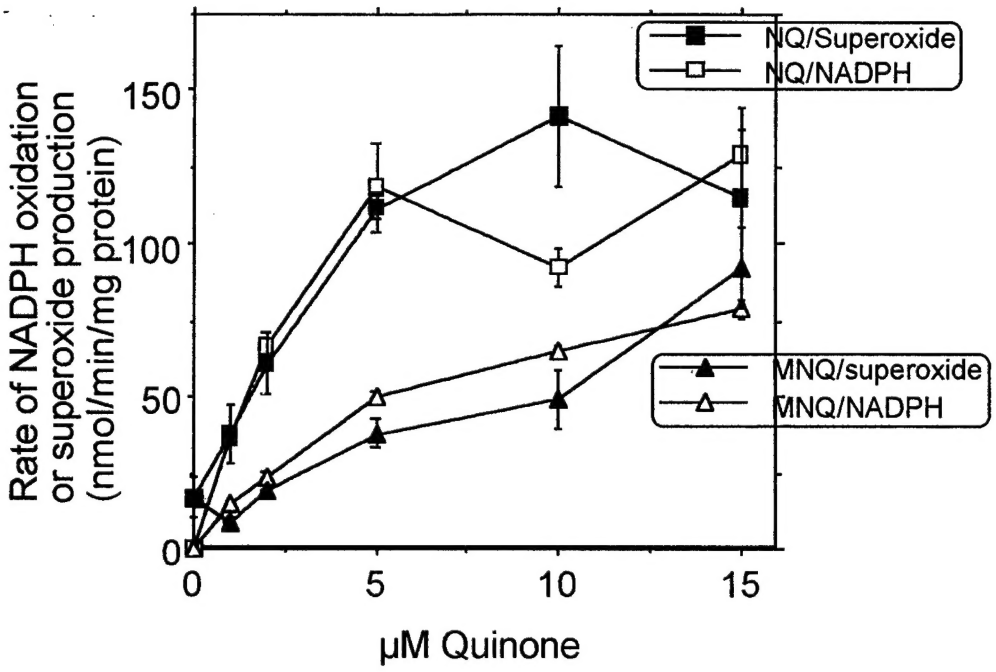

Fig. 12. Effects of adding increasing amounts of NQ or MNQ on the oxidation of NADPH and the production of superoxide by hepatic microsomes from human N (0.135 mg) under aerobic conditions. Points represent the mean for $n = 2$, and bars represent the range of high and low values; for points lacking apparent range bars, the bars were smaller than the points as shown.

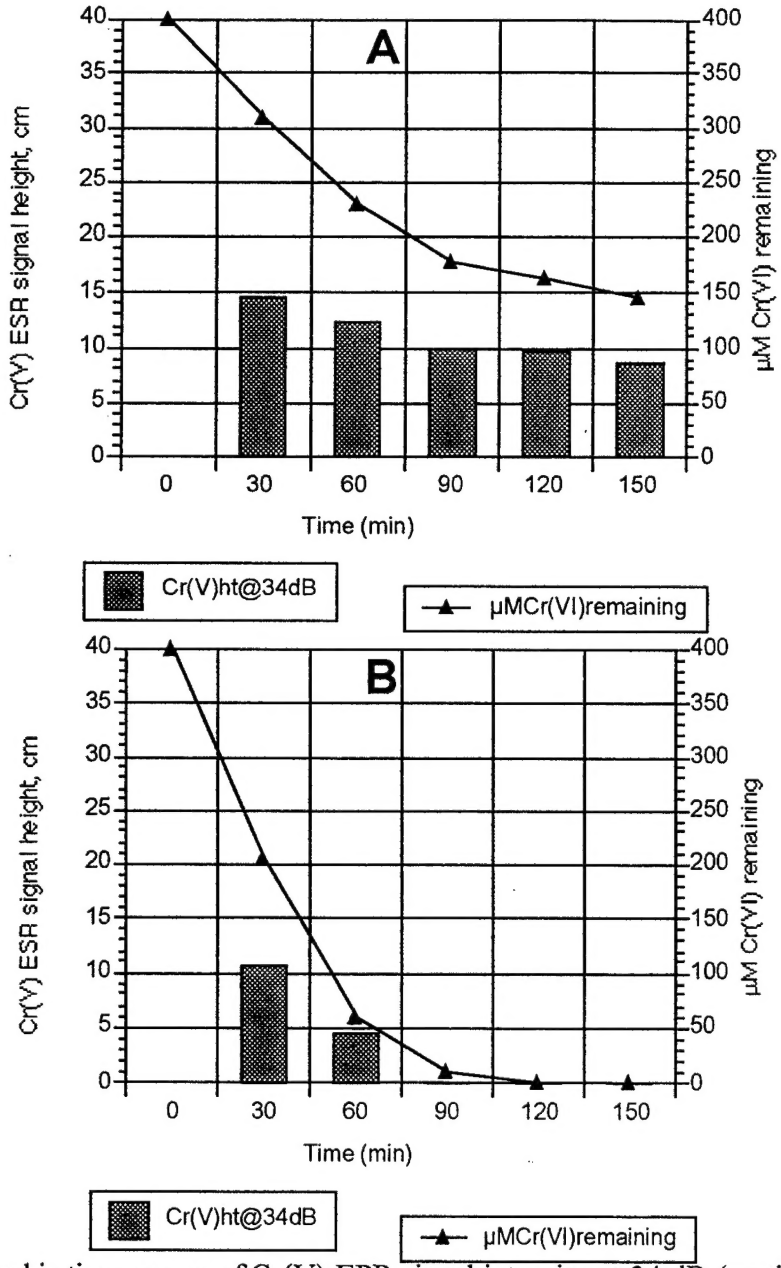

Fig. 13. Anaerobic time course of Cr(V) EPR signal intensity at 34 dB (vertical bars) relative to the level of Cr(VI) remaining (▲), using human Q lung microsomes (1.5 mg), 400 µM initial Cr(VI), and an NADPH-generating system. **A**: active microsomes plus low-iron NADPH-generating system; **B**: same as A but incl. 10 µM NQ. Samples were analyzed at 30-min intervals over the 150-min time course. A portion of each sample was frozen (77 K) for EPR analysis, while the rest was processed for Cr(VI) analysis. EPR instrument settings were the same as Fig. 3.

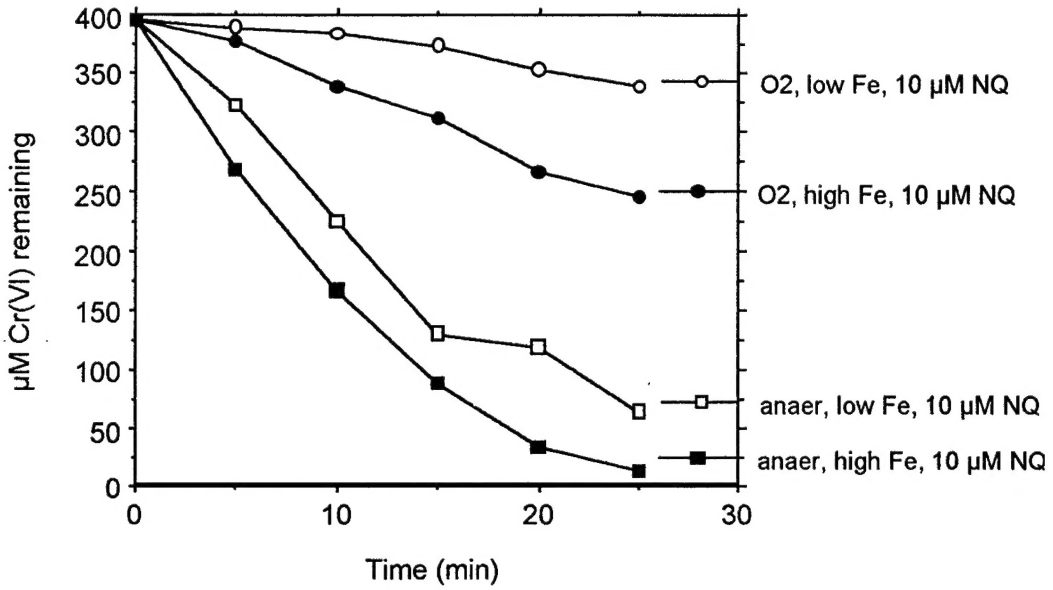

Fig. 14. Effects on NADPH-dependent Cr(VI) reduction of adding 13 µM $FeCl_3$ (high iron) to human N hepatic microsomes (1.56 mg) in the presence of 10 µM NQ. O_2 is room air, and anaer. is no O_2. Points represent the conc. of Cr(VI) remaining at each time point.